Anti-inflammatory and Cytotoxic Chemical Constituents from the Trunks of *Berberis wallichiana*

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The chemical compositions and biological activities of the *Berberis wallichiana* trunk were evaluated for the first time. The alkaloid berberine was found as the main constituent of this plant. In the essential oil fraction, safrole was the most abundant component. The isolated 8-oxypalmatine compound exhibited cytotoxic effects on all three cancer cell lines A549 (human lung carcinoma), MDA-MB-231 (human breast carcinoma), and DU145 (human prostate carcinoma), while berberine was the most active to DU145 cells (IC_{50} of 4.99 μM). This alkaloid compound also potently inhibited the production of nitric oxide with IC_{50} at 0.017 μM. These findings suggested that the trunks of *B. wallichiana* might be a good source of bioactive compounds.

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INTRODUCTION

The Berberidaceae family comprises 17 genera and about 650 species, and is distributed mainly in the Northern Hemisphere, originating from warm temperature regions, including Vietnam. Among the family, the genus *Berberis* L. is the most abundant, with approximately 500 species. In Vietnam, six *Berberis* species including *B. hypoxantha*, *B. kawakami*, *B. julianae*, *B. sargentiana*, *B. subacuminata*, and *B. wallichiana* have been recorded to be distributed mainly in the limestone mountainous areas of the northern provinces. Trunks and roots of *B. wallichiana* and other *Berberis* species have been used in traditional medicine to heal wounds, diarrhea, fever, eye diseases, jaundice, pregnancy vomiting, rheumatism, kidney stones, and gallstones (Chi 2004). The main chemical constituents of the species in the genus *Berberis* are alkaloids, steroids, glycosides, flavonoids, saponins, terpenoids, and reducing sugars (Khan *et al.* 2016). Secondary metabolites and alkaloids (most importantly berberine) from these species have antibacterial, antifungal, antiviral, antidiabetic, and anti-tumor properties (Brückner 2000; Imenshahidi and Hosseinzadeh 2016; Ali Redha *et al.* 2021). In Vietnam, the trunks and roots of *Berberis wallichiana* have been overexploited for treatment of diarrhea, trachoma, skin inflammation, osteoarthritis, *etc.* (Ngo *et al*. 2017). To the authors' best knowledge, though trunks of *B. wallichiana* have been used widely in traditional medicine, their chemical compositions and biological activities have not yet been reported. In this study,

the extract and fractions of *B. wallichiana* trunks showed remarkable cytotoxicity against several cancer cell lines and exhibited strong inhibition of the production of anti-inflammatory mediator nitric oxide (NO). This paper describes the extraction and identification of the chemical constituents as well as the cytotoxic and anti-inflammatory effects of this species.

EXPERIMENTAL

Plant Materials

The trunks of *Berberis wallichiana* were collected from Lao Cai province, Vietnam in Jun 2016. The sample was identified by one of the authors (Bui Van Thanh), and a voucher specimen (M-186.2016) was deposited at the herbarium of the Institute of Ecology and Biological Resources, Vietnam.

General Procedures

Thin-layer chromatography (TLC) was performed on precoated silica gel 60 F254 plates (Merck, Darmstadt, Germany), and the spots were detected under UV illumination at wavelengths of 254 nm and 365 nm, followed by spraying with 10% H_2SO_4 reagent and heating the plates. Column chromatography (CC) was performed using Diaion HP-20 (Mitsubishi Chem. Ind. Co., Ltd., Chiyoda, Japan), silica gel 60 (70- to 230-mesh, Merck, Darmstadt, Germany), or YMC RP-C18 resin (150 μm, YMC, Kyoto, Japan). Nuclear magnetic resonance (NMR) experiments were performed on a Bruker AM500 Fourier transform NMR (FT-NMR) spectrometer (Bruker, Billerica, MA, USA) using tetramethylsilane (TMS) as an internal standard. Optical rotations were read on a JASCO P-2000 digital polarimeter (JASCO Corporation, Tokyo, Japan). Electron-spray ionization (ESI) mass spectra were obtained with an Agilent 1260 series Liquid chromatography-mass spectrometry (LC-MS) single quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA).

Essential Oil Analysis

Dried *B. wallichiana* trunk powder (500 g) was hydrodistilled in a Clevenger-type apparatus for 4 h, after which the essential oils obtained were separated and dried with anhydrous $MgSO_4$. The obtained oils were stored at -5 °C until used. The gas chromatography-mass spectrometry (GC-MS) analysis was performed using an Agilent GC7890A apparatus coupled to a mass selective detector (Agilent 5975C; Agilent Technologies, Santa Clara, CA, USA). An HP-5MS fused silica capillary column (60 m × 0.25 mm id. × 0.25 μm film thickness) was used. Helium was the carrier gas with a flow rate of 1.0 mL/min. The inlet temperature was 240 °C and the oven temperature program was as follows: 60 °C to 220 °C at 4 °C/min, and then at 20 °C/min to 240 °C with an inter-phase temperature of 280 °C. The split injection mode was 1:142, the detector temperature used was 270 °C, and the injection volume was 0.1 μL. The MS interface temperature was 270 °C, MS mode, E.I. detector voltage 1300 V, and mass range 40 Da to 400 Da at 1.0 scan/s. Identification of components was achieved based on their retention indices and by comparison of their mass spectral fragmentation patterns with those stored in the MS library (NIST08, Wiley09). Relative contents of the various chemical components were calculated based on total ion current without standardization. Data processing was achieved using MassFinder4.0 (Hochmuth Scientific Consulting, version 4.0, Hamburg, Germany).

Extraction and Isolation

Dried trunk powder (5 kg) was extracted with methanol (MeOH) (15 L × 4 times) in an ultrasonic bath for 45 min. The combined solutions were concentrated to obtain a crude extract (100 g). The extract was suspended in 10% HCl solution, then successively partitioned three times in ethyl acetate (EtOAc) at the ratio of 1:1 (v/v). The organic layer was separated and concentrated to give the non-alkaloid fraction (6.1 g), while the acid phase was alkalinized to pH 8 to 9 with a 2 N NaOH solution then extracted four times with the two-fold volume of dichloromethane. The organic layer was concentrated to give the alkaloids enriched fraction (20.0 g). The enriched fraction was subjected to a silica gel chromatography column (CC) with gradient mixtures of dichloromethane (DCM) and MeOH (60:1 to 1:1, v/v) to afford six fractions (F1 to F6). Berberine (**1**) (4.8 g) obtained from the fraction F6 (10 g) was further purified by recrystallization in MeOH. Fraction F3 (374 mg) was subjected to a silica gel CC and eluted with a solvent mixture DCM-EtOAc-Acetone (60:1:1, v/v/v), followed by a YMC-C18 column eluted with MeOH 60% to obtain noroxyhydrastine (**2**) (4.4 mg) and 8-oxypalmatine (**3**) (8 mg). Fraction F2 (250 mg) was separated using a silica gel column eluted with DCM-EtOAc (16/1, v/v) followed by a silica gel column eluted with DCM-acetone (40/1, v/v) to afford oxyberberine (**4**) (80 mg) and berberal (**5**) (4 mg).

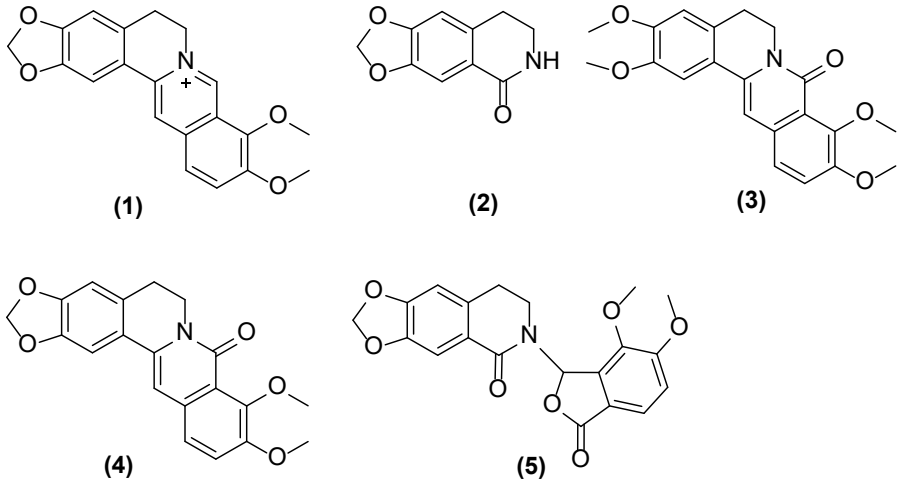

Fig. 1. Structures of the isolated compounds **1** to **5**

Cell Lines

The RAW 264.7 (Murine macrophage), A549 (human lung carcinoma), MDA-MB-231 (human breast carcinoma), and DU145 (human prostate carcinoma) cells were kindly provided by Prof. Jeong-Hyung Lee, Department of Biochemistry, College of Natural Sciences, Kangwon National University, Korea. The cells were cultured at 37 °C in an RMPI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin sulfate, and 100 U/mL penicillin. The cells were incubated in a 5% CO_2 incubator.

Cytotoxicity Assay

The viability of the cells was evaluated using the methylthialazole tetrazolium bromide (MTT) method. The cells were seeded in 96-well plates at the concentration of 1.0×10^5 cells/well and treated with various concentrations of isolated compounds, then

incubated in a humidified 5% CO_2 atmosphere at 37 °C. After 48 h incubation, 20 µL of MTT (0.5 mg/mL in PBS) was added to each well and incubated again at 37 °C for 4 h. After removing the supernatant, the solids obtained were dissolved in isopropanol, and the OD values were measured at 570 nm using a microplate reader. Camptothecin was used as the positive control for the tests on A549 and DU145 cell lines, whereas paclitaxel was used as the positive control for the test on MDA-MB-231 human breast carcinoma cells. Camptothecin is an antitumor agent that has been used not only as a popular positive control on cytotoxicity assays (Li *et al*. 2017; Qin *et al.* 2017; Hue *et al.* 2022). Meanwhile, paclitaxel is also a well-known drug for breast cancer treatments. Measurements were performed in triplicate and are representative of two independent experiments in which the values generally agreed within 5%.

Assay for Inhibition of NO Production

RAW264.7 cells were seeded in a 96-well plate at 2.5×10^5 cells/well and incubated for 3 h. The plates were pretreated with various concentrations of compounds for 30 min and then incubated for another 24 h with or without 1.0 µg/mL lipopolysaccharide (LPS). As a parameter of NO synthesis, nitrite concentration in the culture supernatant was measured by the Griess method. A total of 100 µL of the culture supernatant was transferred to another 96-well plate and mixed with 100 µL of Griess reagent. The absorbance of the reaction solution was read at 570 nm with a microplate reader. Cardamonin was used as a positive control. Measurements were performed in triplicate and are representative of two independent experiments in which the values generally agreed within 5%.

RESULTS AND DISCUSSION

Essential Oil Chemistry

The chemical compositions of essential oil obtained from *B. wallichiana* were analyzed by GC/MS. As shown in Table 1, a total of 53 components amounting to 93.3% were identified. Safrole was found as the most abundant (22.28%), while *α*-cedrene, (E)-caryophyllene, cedrol, and *α*-cadinol were the major components (accounted from 2.06% to 4.91%). Another major compound at 15.89% content level was recognized but the chemical name has not been identified from the MS libraries.

Structures Elucidation

Five alkaloids were isolated from the extract of *B. wallichiana* trunks. Berberine (**1**) was recrystallized and identified by comparing on TLC and LC-MS experiments with the in-house standard compound. This is a popular alkaloid found in several species of the *Berberis* genus (Bruce *et al.* 1987). The ^{1}H-NMR spectrum was used to further confirm the structure of the compound.

Compound **2** was isolated as a pale yellow amorphous powder. The ^{1}H-NMR spectrum showed two singlet signals for the aromatic ring at δ_H 7.52 and 6.65 ppm, a triplet of a methylene group at δ_H 2.93 ppm, a triple doublet of methylene at δ_H 3.52 ppm, and a broad singlet of amide proton at δ_H 6.13 ppm suggested their characteristic linkages. The ^{13}C NMR spectrum showed signals of two methylene, two aromatic methines, four quarternary aromatic carbons, an amide carbonyl group, and a methylenedioxy group. Comparison of NMR spectra of compound **2** with reported data indicated it to be noroxyhydrastine (Bhakuni and Singh 1982).

Table 1. Essential Oil Composition of *B. wallichiana*

No.	Retention Time (min)	Retention Index	Compound Name	Area (%)
1	17.66	1157	Citronellal	0.32
2	20.30	1232	Citronellol	1.41
3	21.24	1259	Geraniol	0.23
4	22.67	1301	Safrole	22.28
5	25.66	1391	*α*-Copaene	0.60
6	26.13	1406	*cis*-*β*-Elemene	0.83
7	26.31	1411	*α*-Methyleugenol	0.53
8	27.02	1434	*α*-Cedrene	3.33
9	27.19	1439	*(E)*-Caryophylene	4.91
10	27.32	1443	*β*-Cedrene	0.47
11	27.46	1448	*α*-*trans*-Bergamotene	0.26
12	27.61	1453	*cis*-Thujopsene	0.34
13	27.82	1459	Neryl acetate	1.63
14	28.09	1468	*β*-Barbatene	1.32
15	28.29	1474	*α*-Humulene	0.82
16	28.51	1481	9-*epi*-*(E)*-Caryophyllene	0.31
17	28.89	1493	*γ*-Muurolene	0.40
18	28.94	1495	ar-Curcumene	0.27
19	29.08	1499	*(E)*-*β*-Ionone	0.37
20	29.13	1501	Germacrene D	0.45
21	29.33	1508	*β*-Selinene	0.30
22	29.43	1511	Asaricin	1.92
23	29.60	1517	*α*-Muurolene	1.37
24	29.72	1521	*β*-Bisabolene	1.30
25	29.83	1524	*β*-Methyl ionone	0.18
26	29.93	1528	Cuparene	0.38
27	30.10	1534	*γ*-Cadinene	0.43
28	30.30	1540	*δ*-Cadinene	1.53
29	30.37	1543	*cis*-Calamenene	0.56
30	31.02	1565	*α*-Calacorene	0.23
31	31.15	1569	Elemol	0.83
32	31.34	1575	*(E)*-*N*erolidol	1.07
33	32.17	1603	Spathulenol	1.84
34	32.35	1610	Caryophyllene oxide	1.49
35	32.89	1629	*epi*-Cedrol	0.51
36	33.02	1633	Cedrol	4.49
37	33.58	1653	*cis*-Cadin-4-en-7-ol	0.41
38	33.72	1658	γ-Eudesmol	1.01
39	33.95	1666	*epi*-*α*-Cadinol	1.00
40	33.99	1668	*epi*-*α*-Muurolol	0.94
41	34.08	1671	*α*-Muurolol	0.60
42	34.36	1681	*α*-Cadinol	2.35
43	34.97	1702	Cadalene	0.23
44	35.06	1705	*epi*-*α*-Bisabolol	0.29
45	35.14	1708	*n*-Heptadecane	1.21
46	37.98	1815	*cis*-5-Hydroxycalamenene	2.06
47	39.33	1868	Unknown	15.89
48	40.07	1897	Unknown	1.16
49	41.41	1951	Unknown	1.32
50	41.57	1957	Methyl palmitate	0.32
51	42.23	1985	Isophytol	0.72

No.	Retention Time (min)	Retention Index	Compound Name	Area (%)
52	42.67	2003	Unknown	1.34
53	47.12	2190	Unknown	2.95
Total				93.31

The NMR spectra of compounds **3** and **4** were almost similar to the data of **1**. These indicated the structures of two protoberberine alkaloids. In these two compounds, the signal of the aromatic methine at C-8 was changed to a signal of a carbonyl group. Moreover, the spectra of compound **3** displayed the disappearance of the methylenedioxy signal and revealed two more methoxy groups. These were identified as 8-oxypalmatine **(3)** and oxyberberine **(4)** by comparing them to the previous study (Paulo *et al.* 1992).

Compound **5** was obtained as a yellow amorphous powder. The ^{1}H and ^{13}C NMR displayed two signals of the aromatic ring and two signals of methylene groups, which were similar to compound **2**. However, the disappearance of amide proton, together with the appearance of a signal of a highly oxidized sp^3 methine (at δ_C 89.7 ppm), signals of an aromatic ring, and two methoxy groups revealed the substitution in the amide group. Comparison of NMR spectra of compound **5** with the reported data indicated it to be berberal (Karimov *et al.* 1993).

Biological Activities

Cytotoxic activities on three cancer cell lines A549 (human lung carcinoma), MDA-MB-231 (human breast carcinoma), and DU145 (human prostate carcinoma) of the isolated compounds were performed and the results are shown in Table 2.

Table 2. IC_{50} Values of Cytotoxicity and Inhibition of NO Production of the Tested Samples

Samples	A549	MDA-MB-231	DU145	NO Production
Total Extract [a]	> 50	> 50	> 50	20.71 ± 3.17
Essential Oil [a]	> 50	> 50	> 50	7.45 ± 1.58
Alkaloids Enrichment [a]	42.21 ± 2.15	> 50	>50	6.31 ± 1.13
Non-alkaloid Fraction [a]	> 50	> 50	>50	5.28 ± 0.78
Berberine (**1**) [b]	> 30	> 30	4.99 ± 0.29	0.017 ± 0.008
Noroxyhydrastine (**2**) [b]	> 30	> 30	> 30	3.48 ± 0.56
8-Oxypalmatine (**3**) [b]	7.22 ± 0.34	16.63 ± 1.38	20.35 ± 1.32	6.1 ± 1.01
Oxyberberine (**4**) [b]	> 30	> 30	> 30	1.41 ± 0.23
Berberal (**5**) [b]	> 30	> 30	> 30	>30
Camptothecin*	12.56 ± 1.45	-	0.007 ± 0.001	-
Paclitaxel*	-	4.74 ± 0.56	-	-
Cardamonin*	-	-	-	1.17 ± 0.31

[a] IC_{50} in µg/mL; [b] IC_{50} in µM; * Positive controls; The values are mean ± SD (n=3, $p<0.05$)

Among five isolated compounds, 8-oxypalmatine exhibited potent effects on all three cancer cell lines, especially on A549 with IC_{50} at 7.22 μM, which was lower than the value of positive control. Meanwhile, berberine displayed the strongest cytotoxic effect on human prostate carcinoma cells DUI145 with IC_{50} at 4.99 μM. The other compounds had no remarkable toxicity on three cancer cell lines. The essential oil of *B. wallichiana* trunks showed no activity against all three cell lines.

The inhibition tendencies of the isolated compounds on the NO production from LPS stimulated RAW 264.7 macrophages cells were evaluated by the Griess method as described above. The result indicated that berberine (**1**) exhibited the strongest inhibition with IC_{50} at 0.017 μM, followed by oxyberberine (**4**), noroxyhydrastine (**2**), and 8-oxypalmatine (**3**) with IC_{50} at 1.41, 3.48, and 6.10 μM, respectively, while the value of the positive control (Cardamonin) was 1.17 μM. By comparison, berberal exhibited weak NO inhibition, with $IC_{50} > 30$ μM. Meanwhile, both alkaloids enriched and the non-alkaloids fraction exhibited remarkable activity with IC_{50} at 5.28 to 6.31 μg/mL, which were much higher than the activity of the total extract. The essential oil also showed remarkable activity with IC_{50} 7.45 μg/mL. The MTT assay showed that the compounds had no remarkable toxicity at their effective doses for the NO inhibition (data not shown).

Discussion

Though a lot of investigations about the chemical compositions of *Berberis* species have been conducted, few of these focused on the essential oils. Previously, Hashemi-Moghaddam *et al.* (2018) reported 18 isolated compounds from roots of *Berberis intergerrima* and found that fatty acids (hexadecanoic acid, linoleic acid, and oleic acid) were major constituents. By contrast, this study reported for the first time over 50 components of essential oils from *B. wallichiana* roots that contributed sharply to the knowledge about chemical compositions of the plant, as well as the genus. Safrole, a phenylpropanoid derivative, was determined as the most abundant at 22.28%, while several terpenoids, such as *α*-cedrene, *(E)*-caryophyllene, cedrol, and *α*-cadinol were present at 2% to 4% levels of the essential oil compositions. Considerably, the second-highest component of the essential oil component was unidentified. Moreover, the essential oil components *B. wallichiana* exhibited remarkable NO inhibition activity (Table 2). Thus, there should be further research on *B. wallichiana* essential oils.

The phytochemical investigation of *B. wallichiana* root led to the isolation of five alkaloids. These compounds were also determined in other *Berberis* species as reported previously, with considerable bioactivities such as antitumors, antimicrobial, and anti-inflammation activities (Karimov *et al.* 1993; Mokhber-Dezfuli *et al.* 2014; Imenshahidi and Hosseinzadeh 2016; Khan *et al.* 2016). The present study investigated anticancer activities of *B. wallichiana* total extract, non-alkaloid fraction, and alkaloids enriched fraction, together with the isolated compounds. As shown in Table 2, berberine performed the strongest inhibition on DU145, followed by oxyberberine (**4**) and noroxyhydrastine (**2**), while showing weak cytotoxicities on the other tumor cells. Meanwhile, 8-oxypalmatine (**3**) exhibited medium inhibition on all three tumor cell lines. The total extract, alkaloids enriched fraction, and non-alkaloid fraction also showed weak anticancer activities. In contrast, the anti-inflammatory activities of the fractions and compounds were also evaluated. As shown in Table 2, except for berberal showing weak activity, the other four compounds exhibited remarkable NO-producing inhibition. Considerably, the anti-inflammatory activity of berberine (**1**) (IC_{50} 0.017 μM) was much better than the positive control, cardamonin (IC_{50} 1.17 μM). Moreover, the alkaloid enriched and non-alkaloid

fractions also inhibited NO production dramatically with IC_{50} at approximately 6 µg/mL. The anti-inflammation of the alkaloids enriched fraction could be contributed by the remarkable activities of the isolated compounds. Benzylisoquinoline alkaloids were investigated from many *Berberis* species with considerable anti-inflammatory and antitumor effects. However, non-alkaloid constituents were not commonly found and evaluated bioactivities from these species. In this study, the non-alkaloid fraction exhibited promising anti-inflammatory activity. While no previous phytochemical investigation has been reported, further investigations should be conducted to identify the chemical compositions of this fraction.

CONCLUSIONS

1. For the first time, phytochemical compositions of *B. wallichiana*, including those of essential oils and alkaloids, were reported. Bioactivities of essential oils, alkaloids, and non-alkaloid fractions from the species were also evaluated.
2. Fifty three chemical compounds, which amounted to 93.3% of the total composition, were determined from essential oils of *B. wallichiana.* Safrole was found as the most abundant, followed by *α*-cedrene, (E)-caryophyllene, cedrol, and *α*-cadinol. Additionally, an unidentified compound was found as a major component (15.9%) that could be a promising target for further investigation of *B. wallichiana* essential oils.
3. Five alkaloids were isolated from roots of *B. wallichiana*, of which, berberine exhibited remarkable inhibition of DU145 and anti-inflammation. Meanwhile, 8-oxypalmatine showed both anti-tumor and anti-inflammatory activities. Moreover, the alkaloids enriched and the non-alkaloids fractions from roots of *B. wallichiana* also performed dramatic NO producing inhibition.
4. In conclusion, this study suggested that the trunks of *B. wallichiana* might be a good source of bioactive compounds. The conservation and sustainable use is needed to prevent the overexploitation of this plant.

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